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(54) Title: METHODS FOR INDUCING SELECTED CELL TYPES

(57) Abstract

The present invention provides methods of using instructive or permissive induction to induce selected cell types in an animal. Usually, the methods involve direct implantation of mesenchyme into suitably prepared sites of adult animals. As a result, new functional organs are created *in situ* by mesenchymal induction of adult epithelial cells. Thus, the methods of the invention can be used in place of organ transplants in patients with chronic or irreversible organ failure. The invention also provides models for studying cancer and methods of reducing tumorigenicity. Cells, tissue recombinants and kits are provided.

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METHODS FOR INDUCING SELECTED CELL TYPES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of provisional application USSN 60/003,735 filed September 14, 1995 by Lipschutz *et al.*, entitled "Methods for Inducing Selected Cell Types."

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under grant No. DK32157, awarded by the National Institutes of Health. The government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to the regeneration of tissue. In particular, it relates to the use of mesenchymal cells to induce differentiation in terminally differentiated adult tissues, thereby producing selected cells or tissue.

BACKGROUND OF THE INVENTION

Mesenchyme is embryonic connective tissue involved in the development of the integumental, gastrointestinal, skeletal, and urogenital systems. Mesenchyme induces a broad spectrum of epithelial forms, including simple epithelial ducts and tubules; branched ductal networks; planar epithelial surfaces and unique epithelial patterns such as those found in teeth, feathers and sense organs (Wessells, N.K. *Tissue interactions and development* (1977) 276; Sawyer, R.H. *In Epithelial-Mesenchymal Interactions in Development* (1983) 115-146; Cunha, G.R. *et al. J. Andrology* (1992) **13**:465-475). Evidence suggests that during these morphogenetic processes, epithelial proliferation is regulated by the mesenchyme (Alescio, T., *et al. J. Exp. Zool.* (1962) **150**: 83-94; Alescio, T. *et al. J. Embryol. Exp. Morphol.* (1967) **17**:213-227; Aaronson, S.A. *et al. Am. Rev. Respir. Dis.* (1990) **142**:S7-S10; Cunha, G.R. *et al. In Sex Hormones and Antihormones in Endocrine Dependent Pathology: Basic and Clinical Aspects* (1994) 45-57). Mesenchyme-induced epithelial development culminates in the emergence of specific types of epithelial cytodifferentiation and the expression of tissue-specific macromolecules (Rutter, W.J. *et al. J. Cell. Physiol.* (1968) 72, Suppl. 1: 118; Higgins, S.J. *et al. Development* (1989) 106: 235-250; Haffen, K. *et al. Differentiation* (1983) **23**:226-233; Donjacour, A.A. *et al. Endocrinology* (1993) **131**: 2342-2350.)

Many organs form as a result of mesenchymal-epithelial interactions. For instance, the kidney forms as a result of interactions between the mesenchymal metanephric mesoderm and the epithelial ureteric bud (which also forms the ureter) (Saxen, L. *Organogenesis of the kidney* (1987) Cambridge University Press, New York).

Mesenchymal-epithelial tissue recombinants can be made in which the mesenchyme induces the epithelium in a permissive or instructive manner. Tissue interactions characterized as permissive inductions are those in which mesenchyme permits the epithelium to express its normal developmental fate. Instructive inductions are those in which mesenchyme induces an epithelium to express an entirely new developmental fate specified by the mesenchyme.

In known instructive inductions, the epithelium is not irreversibly committed and, therefore, retains the ability to respond to a heterologous mesenchymal inductor. For example, in the presence of androgens, urogenital sinus mesenchyme can elicit prostatic development in epithelia of the female urogenital sinus, postnatal vagina, and fetal or postnatal bladder (Cunha, G.R. *et al. J. Exp. Zool.* (1978) 205: 181-194; Cunha, G.R. *Anat. Rec.* (1972) 172: 179-196; Boutin, E.L. *et al. Differentiation* (1991) **48**: 99-105; Cunha, G.R. *Endocrinology* (1975) 95: 665-673; Cunha, G.R. *et al. J. Cell Biol.* (1983) 96: 1662-1670). It should be noted that all these epithelia are derived in total or part from the embryonic urogenital sinus (Cunha, G.R. *In Urologic Endocrinology* (1986) 6-16). The epithelial ducts induced by the urogenital sinus mesenchyme express histological, ultrastructural, and functional features indicative of prostate: Androgen receptors, prostate-specific antigens; androgen dependency for DNA synthesis; and prostatic secretory proteins (Cunha, G.R. *et al. J. Cell Biol.* (1983) 96: 1662-1670; Cunha, G.R. *et al. Biol. Reprod.* (1980) **22**: 19-43; Cunha, G.R. *et al. Endocrinology* (1980) 107: 1767-1770; Neubauer, B.L. *et al. J. Cell Biol.* (1983) 96: 1671-1676). None of these changes occur if the epithelium is grown by itself (Cunha, G.R. *Int. Rev. Cytol.* (1976) **47**: 137-194; Cunha, G.R. *et al. J. Cell Biol.* (1983) 96: 1662-1670).

Both instructive and permissive inductions by urogenital mesenchyme are mediated by similar signals in a variety of mammalian species, as heterospecific prostatic inductions have been observed in tissue recombinants constructed of mixtures of mouse, rat, rabbit, and human tissues (Cunha, G.R. *et al. Differentiation* (1983) **24**: 174-180).

The adult counterpart of embryonic mesenchyme is stroma, which constitutes the non-epithelial component of an organ. The predominant cells of the stroma

are fibroblasts and smooth muscle. The role of stromal-epithelial interactions has received considerably less attention than that of mesenchymal-epithelial interactions, probably due to the fact that adult epithelial cells were believed to be irreversibly determined and terminally differentiated (Ursprung, H. *The Stability of the Differentiated State* (1968) Springer-Verlag, NY; Slack, J.M.W. *From Egg to Embryo: Determinative Events in Early Development* (1985) Cambridge University Press, NY). Nonetheless, there is some evidence suggesting that adult epithelial cells remain responsive to the inductive influences of stromal/mesenchymal cells.

Many examples of developmental plasticity in adulthood involve the stromal induction of regional variation in epidermal differentiation (Bernimoulin, J.P. *et al. Path. Res Pract.* (1980) **166**:290-312; Mackenzie, I.C. *et al. Cell Tissue Res.* (1984) **235**:551-559; Spearman, R.I.C. *Acta Anat.* (1974) **89**:195-202). These findings, however, are best characterized as stromal-induced modulations of epidermal differentiation since minor changes in epidermal thickness and patterns of keratinization are encompassed within the basic stratified squamous epidermal phenotype. In other experimental models, mesenchymal or stromal cells have elicited changes in the morphology, differentiation, or growth of adult epithelial cells (Cunha, G.R. *et al. J. Cell Biol.* (1983) 96: 1662-1670; Dudek, R.W. *et al. Diabetes* (1988) **37**:891-900; Sakakura, T. *et al. Develop. Biol.* (1979) **72**:201-210.) The changes elicited by urogenital sinus mesenchyme in the epithelium of the adult urinary bladder provides one example of a mesenchyme-induced alteration in adult epithelial differentiation. As noted above, in this model, embryonic urogenital sinus mesenchyme induced the epithelial cells of the adult urinary bladder to undergo prostatic differentiation. This resulted in formation of a simple columnar secretory epithelium, morphogenesis of a branched ductal network, marked stimulation in epithelial proliferation, and expression of several prostate-specific markers (Donjacour, A.A. *et al. Endocrinology* (1993) **131**: 2342-2350; Cunha, G.R. *et al. J. Cell Biol.* (1983) 96: 1662-1670; Neubauer, B.L. *et al. J. Cell Biol.* (1983) 96: 1671-1676.)

Seminal vesicle mesenchyme, has been shown to be able to induce seminal vesicle differentiation from adult epithelial derivatives of the Wolffian duct (Cunha, G.R. *et al. Development* (1991) **111**:145-158).

Despite these advances the prior art has not demonstrated that instructive induction can be utilized to regenerate new tissues or organs to replace damaged or diseased tissue *in vivo*. The present invention provides these and other advantages.

SUMMARY OF THE INVENTION

This invention demonstrates that an instructive induction can be achieved *in situ*, *e.g.*, by direct implantation of mesenchyme into suitably prepared sites of adult animals. This invention demonstrates for the first time that new functional organs can be created *in situ* by mesenchymal induction of adult epithelial cells. Thus, the methods of the invention can be used in place of organ transplants in patients with chronic or irreversible organ failure. This invention also demonstrates that differentiation induced by mesenchymal cells can reduce the tumorigenicity of tumor cells.

Accordingly, in one class of embodiments of the invention, methods of producing differentiated cells, tissues and organs *in vitro* and *in vivo*, with a selected phenotype are provided. In the methods, a mesenchymal cell is contacted and incubated with a second cell (typically an epithelial cell). The second cell then produces differentiated cells corresponding to the tissues from which the mesenchymal cells were isolated. Typically the cells differentiate to form tissues or organs. Ordinarily, the mesenchymal cells are selected from cells which direct the production of differentiated cells in the embryo which correspond to the differentiated cells to be produced in the adult (*i.e.*, cells of the selected phenotype). Preferably, this process is performed *in vivo*, producing differentiated tissues or organs *in situ* at the site of mesenchymal cell introduction.

In an alternative embodiment, differentiated cells are removed from an adult animal and cultured *in vitro* in the presence of isolated mesenchymal cells. The resulting differentiated cells are optionally reintroduced into the adult animal where they provide a therapeutic benefit. For instance, where the differentiated cells are pancreatic, the cells are optionally reintroduced into the adult animal at a site which is in contact with the blood stream, so that the cells can produce and deliver insulin to the adult animal. The mesenchymal cell and the second cell are preferably from organisms of the same species, but optionally from organisms of different species.

In preferred embodiments, a plurality of differentiated cells are produced in the adult, thereby forming an epithelial tissue, wherein the tissue is selected from the group of tissues consisting of kidney tissue, pancreatic tissue, bladder tissue, lung tissue, intestinal tissue, liver tissue, and reproductive tissue.

In another group of embodiments, the invention provides methods of producing a selected cell from a terminally differentiated cell, by incubating the terminally

differentiated cell with a mesenchymal cell. Typically the cell is an epithelial cell selected from the group consisting of kidney tissue, pancreatic tissue, bladder tissue, lung tissue, intestinal tissue, liver tissue, and reproductive tissue.

In one particularly preferred embodiment, the invention provides for the regeneration of a defective kidney in an animal by incubating a terminally differentiated ureter cell with kidney mesenchyme. When performed *in vivo*, the ureter cell gives rise to kidney tissue in an anatomically correct arrangement relative to the urinary system.

In one embodiment, a clonal population of cells is used to give rise to a fully differentiated organ. In this embodiment, a primary culture of epithelium is subjected to clonal selection. The clonal cells in culture, which may represent epithelial stem cells, are used in conjunction with mesenchyme to grow a corresponding organ *in vivo*. For example, a single cell (or a population of cells) from a clonal population of cells is grown into a prostate *in vivo* by placing the cell in close proximity to, or contact with, urogenital sinus mesenchyme in a host animal.

The invention also provides kits comprising mesenchymal cells and a container. The kits optionally further comprise terminally differentiated cells, animals, instructional materials and the like.

In a further embodiment, the invention provides cells produced by incubating a terminally differentiated or adult cells with an embryonic mesenchymal cell. These cells are optionally in cell culture or *in vivo*, and are optionally incorporated into tissues or organs.

The invention provides mesenchymal-epithelial tissue recombinants which are models for assessing carcinogenesis and tissue differentiation. The tissue recombinants are a host animal with a mesenchymal cell and an epithelial cell. Often, the host animal, epithelial cell and mesenchymal cell are all from different sources, *e.g.*, the host animal is optionally a mouse (such as an athymic mouse), the mesenchymal cell is optionally from a rat embryo and the epithelial cell is optionally a human cell. In one embodiment, the epithelial cell is a tumor cell.

The tissue recombinants can be used to study the effects of test compounds on differentiation or carcinogenesis. In one embodiment, the test compound is applied to the tissue recombinant host, typically so that the test compound contacts the interacting epithelial-mesenchymal cell. The effect of the test compound on a measurable marker such as differentiation or cell growth are then assessed. The ability to screen test

compounds for their affect on cell growth provides a valuable new model for studying differentiation and tumorigenesis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing illustrating the placement of neonatal rat seminal vesicle mesenchyme on the adult mouse ureter epithelium.

Figure 2 shows an induced mouse seminal vesicle at the severed end of the ureter with the platinum wire visible (small arrow).

DETAILED DESCRIPTION

For purposes of the present invention, the following terms are defined below.

"Phenotype" refers to the detectable physical characteristics of a cell or tissue or organ. A "selected" phenotype is a phenotype which shares physical characteristics with a given known cell, tissue or organ.

As used herein, the term "epithelium", is used to refer to the cellular layer covering all free surfaces in a mammal, including cutaneous, mucous, and serous layers, as well glands and other structures derived from them.

As used herein, the term "mesenchyme" is used to refer to embryonic connective tissue, usually found between the epithelia and tissue masses of major organs.

As used herein, the term "instructive induction' is used to refer to those processes by which mesenchyme induces an epithelium to express an entirely new developmental fate specified by the mesenchyme. These processes are contrasted with tissue interactions characterized as "permissive inductions" in which mesenchyme permits the epithelium to express its normal developmental fate. In the present invention, mesenchymal-epithelial tissue recombinants can be made in which the mesenchyme induces the epithelium in a permissive or instructive manner.

It is generally believed that adult tissue is terminally differentiated. For instance, the ureter is derived from the metanephric diverticulum which, along with the derivatives of the metanephric mesoderm, forms the kidney. In the examples below, the left ureters of adult male athymic mouse hosts were severed below the kidney, and mesenchyme from neonatal rat seminal vesicles (SVM) was grafted to the cut end of the ureter, thus bringing adult mouse ureter epithelium (URE) in contact with neonatal rat SVM. After four to eight weeks, the *in situ* tissue recombinants were harvested, and the epithelial secretory proteins recovered. In 5 of 11 cases, an induction occurred, resulting

in an *in situ* transformation of the non-glandular transitional epithelium of the adult mouse ureter into the simple columnar epithelium of the seminal vesicle (SV). Functional cytodifferentiation was examined in these neonatal rat SVM + adult mouse URE tissue recombinants using antibodies against SV-specific secretory (SVS) proteins of the mouse and rat. From the cut end of the ureter, the adult URE was induced to undergo SV morphogenesis, to express SV cytodifferentiation, and to produce the complete spectrum of major SVS proteins characteristic of the mouse. The induced seminal vesicle epithelium (SVE) also expressed androgen receptors (AR) which are not seen in urothelial tissue. Staining with Hoechst dye 33258, which can distinguish cells of mouse and rat origin, further demonstrated that the induced SVE was indeed of mouse origin and not a contaminant of the inducing rat SVM. In addition, neonatal mouse vaginal mesenchyme was grafted *in situ* beneath the bladder mucosa of adult male mice, and the host animals were killed after 3 months. The vaginal mesenchyme implanted into the bladders induced prostate-like acini, indicating that the above reprogramming of adult organs *in situ* is not an isolated occurrence. These results show that the creation of new vital organs, such as the kidney, are possible *in situ*, by demonstrating that adult epithelial cells retain a developmental plasticity equivalent to their undifferentiated fetal counterparts, and are capable of being reprogrammed *in situ* to express a completely new morphological, biochemical, and functional phenotype.

In addition, cells are permissively reprogrammed to differentiate into functional tissues or organs by placing cells of a selected phenotype (typically an epithelial cell) into contact with mesenchyme which normally directs development of tissues of the same phenotype in an embryo.

This invention demonstrates that an instructive induction can be achieved *in situ* by direct implantation of SVM into suitably prepared sites of adult animals. Since the embryonic Wolffian ducts normally give rise to the epithelia of the epididymis, seminal vesicle, ureter, and ductus deferens, this system provides an excellent model in which the morphological and functional aspects of the reprogramming of adult epithelia can be studied *in situ*, and the results described in the examples below are generally applicable to other organ systems. This invention demonstrates for the first time that new functional organs can be created *in situ* by mesenchymal induction of adult epithelial cells.

In vitro Uses of The Invention

A differentiated cell is a cell with a characteristic morphology or phenotype which expresses a specific set of differentiation markers, *e.g.*, uroplakin for urothelial cells, or PSA for prostate cells. Certain cell types, *e.g.* kidney or liver epithelial cells, are difficult to grow in primary culture. In one embodiment of the present invention, production of tissue-specific factors from these cells is facilitated by incubating embryonic mesenchymal cells associated with the corresponding embryonic tissue type in the presence of more easily isolated differentiated cells such as epithelial cells. By incubating the appropriate mesenchymal cells with terminally differentiated cells to form cultures of desired cell types, the secreted products, gene transcripts, and the cells themselves are more readily available for study and commercial application. Examples of cell products amenable to this type of production are human serum albumin or clotting factors from liver epithelium, steroids from adrenal cortical epithelium and insulin from pancreatic tissue. For genes which are difficult to induce, isolate, or whose mRNA is unstable or exists in low copy numbers, this culture system facilitates gene isolation and cloning. Experimental model systems for studying cellular and organ drug metabolism, fluid and electrolyte transport, or secretory mechanisms are improved by the co-culture of specific epithelia and mesenchymal combinations which can be manipulated at will, and which, prior to the present invention, would not have been readily available to persons skilled in the art.

Generation of Tissues *in vivo*

The present invention provides two general strategies for generating tissues and organs *in vivo*. In the first strategy, the tissues are generated *ex vivo* and transplanted into the animal. In the second strategy, the tissues are generated directly *in vivo*.

Ex vivo Production of Selected Cells and Tissues

In one embodiment of the invention, differentiated tissue from a patient is removed from a relatively accessible site, *e.g.* urethral, bladder or ureter epithelium, and cultured *in vitro* with the appropriate embryonic mesenchyme to instructively induce formation of the tissue type desired, *e.g.*, kidney tubular epithelia, and transplanted back into the donor patient upon redifferentiation *in vitro*. The source of the embryonic inductive mesenchyme is preferably from the animal into which the resulting tissue is to be introduced, *i.e.*, human embryonic mesenchyme is typically used to produce a human

tissue, but alternate mammalian species are also appropriate donors for embryonic mesenchyme.

Because the cells which are induced by the embryonic mesenchyme are isolated from the animal into which the generated tissue is to be introduced, no graft rejection is observed, providing a distinct advantage over donor transplantation, or transgenic xenotransplantation. Because the cells are induced *in vitro*, the source of the inducing agent-- *i.e.*, the embryonic mesenchymal cells-- is not significant from the standpoint of antigenic rejection, or host versus graft rejection.

In one example, epithelial tissue from the rectum would be obtained from a patient suffering from ulcerative colitis, and cultured with embryonic mesenchyme from the gut *in vitro*, to produce intestinal tissues which, upon transplantation back into the patient, replace corresponding diseased tissues which are usually removed surgically.

In one embodiment, a clonal population of cells is used to give rise to a fully differentiated organ. In this embodiment, a primary culture of epithelium is subjected to clonal selection. The clonal cells in culture, which may represent stem epithelial cells, are used in conjunction with mesenchyme to grow a corresponding organ *in vivo*. For example, a single cell (or a population of cells) from a clonal population of cells is grown into a prostate *in vivo* by placing the cell in close proximity to, or contact with, urogenital sinus mesenchyme in a host animal. Similarly other clonal epithelial cells (*e.g.*, kidney) can be clonally selected *in vitro* and differentiated into an organ *in vivo*.

Other circumstances for such *ex vivo* production of organs and tissues will be apparent to those skilled in the art, and are contemplated in the present invention.

In Vivo Production of Selected Cells and Tissues

In a preferred embodiment, mesenchyme is placed into a host at a site where tissue, or a replacement organ is to be generated. This strategy provides one of skill with the ability to replace defective organs or tissues *in situ*. Moreover, the replaced organs, because they are ultimately produced by the host, do not encounter the usual problems of tissue rejection associated with standard organ replacement therapy. Thus, the present invention provides for a powerful new method of regenerating damaged organs and tissues.

In the methods of the invention, animals with new tissue derived from the addition of embryonic mesenchyme to the animal are produced by excising the tissue of interest from embryonic, or very young (typically less than 3 days old, preferably less

than 1 day old) neonatal animals and separating the mesenchyme from the tissue by tryptic digestion. For instance, in the examples below, seminal vesicles were excised from 0-day-old neonatal rats and vaginas were excised from 0-day-old neonatal mice. Mesenchymes were separated from epithelium following tryptic digestion as described (Cunha, 0.R. *et al. Human Genetics* (1981) **58**:68-77).

Following overnight culture of the mesenchyme on an appropriate substrate such as 1% agar, the mesenchyme forms into a cohesive mass of tissue which is transplanted into adult animal hosts at a selected site. It is expected that one of skill is familiar with the appropriate surgical techniques for performing tissue transplantation into the host animal. In brief, the animal is anesthetized, the site into which the mesenchyme is to be placed is isolated and surgically exposed, and the mesenchyme is sutured or stapled in place or simply placed in close proximity to the selected site. The surgical field is then closed with sutures or staples or other appropriate means.

Selection of the mesenchyme and the surgical site for transplantation depends on the organ or tissue to be produced. The mesenchyme is isolated from an embryonic or neo-natal organ which corresponds to the organ or tissue to be produced, and the surgical site of implantation of the mesenchyme is selected to provide an appropriate anatomical arrangement for the growing organ or tissue. For instance, a kidney can be induced *in vivo* by transplanting human metanephric mesoderm to the animal's ureter. The urinary outflow tract is thus placed in an anatomically correct sequence relative to the developing induced kidney. Competent human metanephric mesoderm is found in embryos around embryonic day 35.

In certain cases, the selected mesenchymal cells may be difficult to obtain or culture *in vitro*. In these cases, techniques for providing a ready supply of the mesenchymal cells is advantageous. For instance, immortalized, temperature sensitive mutants transfected with the gene for the large T antigen of SV40 can be used. These cells are capable of dividing at lower temperature (*e.g.*, about 33°C) but not at body temperature. Thus, the cells will not proliferate in the body, but can be maintained indefinitely at lower temperatures. This illustrates a difference between tumor cells and immortalized cells. Immortalized cells can often be easily controlled by regulation of the factors which make the cell immortal, unlike tumor cells. Immortalized cells can be constituively or inducibly immortalized.

The procedures described herein are generally applicable to all mammals, including rodents such as mice, rats, and guinea pigs, primates such as monkeys, baboons, macaques, chimpanzees, gorillas, and especially humans, dogs, cats, sheep, cattle, goats, horses *etc.* It is generally preferred that the isolated mesenchyme be isolated from an embryo of the same species into which the organ or tissue is to be induced in order to reduce complications from tissue rejection. However, because of the similarity of the signals which induce tissue differentiation across the mammals, the source of mesenchyme is optionally from an animal species different from the animal into which the mesenchyme is to be placed. Thus, for instance, rat mesenchyme can be used to induce tissue or organ formation in mice. Similarly, rat mesenchyme can induce tissue formation with human epithelial cells in mice. Where mesenchyme is used from allogeneic or xenographic source, the animal into which the mesenchyme is to be placed is typically put on a regimen of immunosuppressants to inhibit tissue rejection. Alternatively, methods of preventing access of the host animals immune system can be used to prevent allograft rejection. For example, artificial pancreatic devices containing live islets have been designed to avoid immune rejection, by enclosing islets in a semipermeable pouch or matrix which separates the transplanted islets from immunoreactive cells and molecules (*see, e.g.*, Newgard, U.S. Patent No. 5,427,940 and Bae, et. al., U.S. Patent No. 5,262,055). Such methods can be used for preventing immune response against mesenchymal cells, as well. Certain animals are genetically engineered to be immune suppressed (*e.g.*, nude mice), or to have immune systems derived from other animal species (*e.g.*, mice expressing human immune genes).

In the examples below, rat mesenchyme was placed into immune compromised mice (*e.g.*, "athymic" mice) which did not require immuno-suppression. In some embodiments, transgenic animals which have the same surface antigens as the host into which the mesenchyme is placed are used as mesenchyme donors to alleviate complications due to tissue rejection.

Primary Culture

Techniques and methods for establishing a primary culture of cells for use in the *in vitro* and *ex vivo* methods of the invention are known to those of skill in the art. Freshney (*Culture of Animal Cells, a Manual of Basic Technique, third edition* Wiley-Liss, New York (1994)) and the references cited therein, Humason (*Animal Tissue Techniques, fourth edition* W.H. Freeman and Company (1979)) and Ricciardelli, *et al.*,

In Vitro Cell Dev. Biol. **25**:1016-1024 (1989) provide a basic guide to tissue culture techniques. Variations in growth media, use of sera, media supplements, culture chambers and substrates are typically determined empirically, depending on the particular cells being cultured and the use for which the cells are harvested.

The growth medium for primary cell culture will typically be a buffered salt solution containing amino acids, vitamins and optionally includes other nutrients, or serum, or a serum-substitute. Well known Examples of such growth media are DMEM and RPMI. Additionally, the medium will contain other supplements to enhance cell growth and prevent the death of the culture.

Buffered salt solutions are designed either to equilibrate with atmospheric conditions or to equilibrate with a gas phase containing 5 to 10% carbon dioxide. In the present inventive methods, solutions of the latter type are preferred. These media are based on Earle's salts and are buffered with a bicarbonate/carbonic acid system which maintains the pH in a CO_2 equilibrated incubator. Well-known example media include Dulbecco's Modified Eagle Medium (DMEM) and RPMI.

Media supplements are optionally added to reduce the need for serum supplementation. Such supplements typically contain growth promoting additives such as insulin, transferrin, trace elements (such as manganese, molybdenum, vanadium, nickel, or tin), ascorbic acid, non-essential amino acids, L-glutamine and other growth factors. Other additives to the growth medium include antibiotics and antifungal agents. Typically, broad spectrum antibiotics such as penicillin, streptomycin, neomycin and polymyxin are used. Preferred antibiotics include penicillin and streptomycin. Preferred antifungal agents are fungizone and nystatin. Particularly preferred is fungizone.

The culture chambers and substrates for primary cells are generally plastic culture dishes or flasks. However, many cells will also grow on glass coverslips placed in plastic dishes and on a variety of membranes and fabrics including collagen membranes, elastic membranes, agar, smooth silicone rubber substrata, polyacrylonitrile fabrics, dacron velour and Parylene-C coated polypropylene microfabric.

The process of trypsinization to remove adherent cells from a culture surface is well known to those of skill in the art. Briefly, trypsin or trypsin•EDTA is solubilized in a Ca^{++} and Mg^{++} free buffered salt solution (*i.e.*, HBSS) and the pH is adjusted to 7.4-7.6. Any media or serum is removed from the monolayer by washing with Ca^{++} and Mg^{++} free buffered salt solution. The trypsin solution is then added to the

vessel containing the monolayer in sufficient quantity to cover the monolayer and the mixture is incubated for about 2 minutes at 37°C. The trypsin solution is removed from the vessel and the monolayer is again incubated until the cells detach from the surface. When this process is complete, serum or medium containing serum is added to the vessel to inhibit further trypsin activity which can damage the cells. These cells can be resuspended by gentle pipetting to break up any clumps, and diluted with media for cell counts and secondary culturing.

When the cultured cells are not used immediately, they are frozen in liquid nitrogen, then thawed and cultured to provide growing cells for further use.

Cell Freezing and Thawing

Cells are optionally frozen to avoid loss by contamination and to provide a constant supply for future use. To freeze cells, cultures are dissociated with trypsin to provide cell pellets which are suspended in complete medium containing either glycerol or dimethylsulfoxide as a cryopreservative. For instance, complete DMEM containing about 10% dimethylsulfoxide (DMSO) is often used. The cell pellet is suspended in the freezing medium at a concentration of about 1 to 5 x 10^7 cells/mL. Aliquots are placed into vials which are cooled to -20°C for 2 hours and then transferred to a liquid nitrogen-containing storage vessel, or -80°C freezer until further use.

Frozen cells are fragile and require gentle handling. Frozen cells should be thawed quickly and are typically plated directly into complete growth media. Cells which are sensitive to the added cryopreservative (*e.g.*, glycerol or DMSO) should be centrifuged, to remove the medium containing the preservative, and then plated into complete growth medium. In a preferred embodiment, vials containing frozen cells are defrosted in a 37°C water bath for one minute. The cells are transferred to a sterile centrifuge tube, complete growth medium is added, and the cells are centrifuged. The supernatant is discarded and the pellet is resuspended in complete medium and plated, *e.g.*, into 75 mm tissue culture flasks to establish a secondary culture.

Secondary Cultures

Secondary cultures can be obtained from previously frozen cells which had become confluent in the primary tissue culture dishes. Centrifugation of the thawed cells provides a pellet which is resuspended in complete medium, counted and plated in tissue culture flasks. Following incubation as described for the primary culture, the cells are typically washed with calcium and magnesium-free phosphate buffered saline solution and

are optionally detached from the dishes mechanically or enzymatically (*e.g.*, by trypsinization).

Detection of Tissue or Organ Formation

The presence of an induced tissue or organ is detected by means well known in the art. In one class of embodiments, the formation of tissues or organs are monitored at the structural level by physical examination (*i.e.*, in conjunction with exploratory surgery), ultrasound examination, or the use of imaging dyes. In a second class of embodiments, the tissues or organs are monitored by detecting the presence of antigens on the surface of the tissues or organs which are specific to the cell types which comprise the organs. This may be done histologically or by monitoring the formation of antigens, *e.g.*, in blood or blood serum. Many tissue-specific dyes, antibodies, and reagents are known in the art.

Measuring Tissue-Specific Markers

The level of tissue-specific markers such as antigens, or mRNA, in a cell culture, cell or whole organism is measured by means known in the art. Typically, the level of marker is measured in a western blot or other immunoassay such as an ELISA, or by performing quantitative PCR on reverse transcribed mRNA. In immunoassay formats, the level of marker is measured by monitoring the amount of a cell epitope or expressed protein by quantifying binding of the protein to an immunogenic reagent such as an antibody. In quantitative PCR, the level of an mRNA is measured by monitoring PCR amplification products, and comparing the amount of amplified nucleic acid obtained, as compared to amplification products obtained from amplification performed on a known reference nucleic acid sample.

Examples of techniques sufficient to direct persons of skill through PCR *in vitro* amplification methods, are found in Mullis *et al.*, (1987) U.S. Patent No. 4,683,202 and *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990).

Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art, and many cell-specific antibodies are commercially available. *See, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY; Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding (1986) *Monoclonal*

Antibodies: Principles and Practice (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) Nature 256: 495-497. Specific monoclonal and polyclonal antibodies and antisera will usually bind a target marker with a K_D of at least about .1 mM, more usually at least about 1 μM, preferably at least about .1 μM or better, and most typically and preferably, .01 μM or better.

Frequently, polypeptides and their corresponding antibodies will be labeled by joining, either covalently or non covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

A particular protein marker can be quantified by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see Stites and Terr (eds.) 1991 *Basic and Clinical Immunology* (7th ed.). Moreover, the immunoassays of the present invention can be performed in any of several configurations, *e.g.*, those reviewed in Maggio (ed.) (1980) *Enzyme Immunoassay* CRC Press, Boca Raton, Florida; Tijan (1985) "Practice and Theory of Enzyme Immunoassays," *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers B.V., Amsterdam; Harlow and Lane, *supra*; Chan (ed.) (1987) *Immunoassay: A Practical Guide* Academic Press, Orlando, FL; Price and Newman (eds.) (1991) *Principles and Practice of Immunoassays* Stockton Press, NY; and Ngo (ed.) (1988) *Non isotopic Immunoassays* Plenum Press, NY.

Cell sorting techniques such as FACS are optionally used to isolate particular cells, *e.g.*, those which express a particular tissue-specific marker from sources such as tissue culture or whole blood.

Antibodies, polypeptides and nucleic acids are detected and quantified by any of a number of means well known to those of skill in the art. These include analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, immunofluorescent microscopy and the like, and various immunological methods such as fluid or gel precipitin reactions,

immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like. The detection of nucleic acids proceeds by well known methods such as Southern analysis, northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography.

Models for Carcinogenesis and Differentiation Therapy

The present invention provides commercially valuable models for studying carcinogenis and a new form of cancer therapy. Cancer is essentially a form of improper cell differentiation. It is now discovered that tumorigenicity of cancer cells can be reduced by causing the cells to undergo differentiation in the presence of normal mesenchyme or stroma from tissue corresponding to a tumor site. This process is referred to as differentiation therapy. Heterospecies tissue recombinants are described which provide a model for assessing tissue development and inhibition of tumors in tissues. For example, a heterospecific prostate model provides for the study of prostate development and tumorigenesis. Furthermore, differentiation therapy was shown to inhibit prostate tumorigenesis *in vivo*

Development, growth and cytodifferentiation of the prostate are androgen-dependent and occur via reciprocal mesenchymal-epithelial interactions. Mesenchymal-epithelial interaction occurs during embryonic periods in which mesenchyme (undifferentiated connective tissue) induces epithelial development, while the epithelium reciprocally induces mesenchymal differentiation. In the developing prostate, urogenital sinus mesenchyme (UGM) acting under the influence of testicular androgens induces ductal morphogenesis, the expression of epithelial androgen receptors (AR), regulates epithelial proliferation and specifies the expression of prostatic lobe-specific secretory proteins. In a reciprocal fashion, the developing prostatic epithelium induces the differentiation and morphological patterning of smooth muscle in the UGM. For example, UGM grown by itself formed minute amounts of smooth muscle. In contrast, when UGM was grown in association with epithelium of either embryonic urogenital sinus, bladder, or adult prostate, prostatic ducts developed which were surrounded by sheathes of actin-positive smooth muscle. The inductive nature of the epithelium was emphasized by the observation that when the epithelium was of rodent origin, the smooth muscle sheathes were thin and thus corresponded to the pattern of rodent prostate. By contrast, when tissue recombinants were prepared with rat UGM plus human prostatic epithelium, the

resultant prostatic ductal tissue was surrounded by thick sheathes of smooth muscle, the pattern characteristic of the human prostate. Thus, the epithelium induces smooth muscle differentiation from the mesenchyme and also specifies its spatial patterning.

A notable feature of prostatic smooth muscle is its dependence upon androgens for development, growth, and maintenance of differentiation in adulthood. These events are presumably dependent upon expression of AR in prostatic smooth muscle. In fetal prostate, smooth muscle α-actin is expressed throughout the UGM, which accounts for the peristaltic contractility of the urogenital sinus in organ culture. During development, smooth muscle differentiation markers appear sequentially, and dense smooth muscle sheathes become organized around the epithelial ducts by 20 days postnatally in the rat. The initial differentiation of prostatic smooth muscle is androgen-dependent both *in vitro* and *in vivo*. This was demonstrated *in vivo* in experiments in which human fetal prostates were divided into right and left halves that were grafted underneath the renal capsule of intact versus castrated male athymic nude mouse hosts. In intact male hosts, prostatic smooth muscle developed extensively throughout the graft. Conversely, in castrated hosts, development of prostatic smooth muscle was impaired. The small amount of smooth muscle observed in such grafts was principally restricted to vasculature. Since prostatic epithelium was present in the human fetal prostate prior to grafting, the paucity of the smooth muscle in grafts to castrated hosts could not be attributed to absence of prostatic epithelium, but instead to an absence of androgenic stimulation. Likewise, in adulthood the maintenance of prostatic smooth muscle in the rat prostate is androgen-dependent. Following castration there is a loss of prostatic smooth muscle as the prostatic smooth muscle bundles disintegrate and disappear with loss of many of the prostatic smooth muscle differentiation markers. Thus, androgen-dependent differentiation of prostatic smooth muscle is dependent upon an interaction with epithelium. Likewise, in adulthood, the maintenance of prostatic smooth muscle is dependent upon androgens and a homeostatic interaction with epithelium.

Prostate tissue is thus formed and maintained by the reciprocal interaction of epithelium and mesenchyme during prostatic development, followed thereafter by a reciprocal homeostatic interaction of epithelium and smooth muscle in adulthood. This smooth muscle-epithelial cell interaction is perturbed during prostatic carcinogenesis with adverse sequelae occurring in both epithelium and smooth muscle. The following sequence of events occurs: (1) Under the influence of androgens, urogenital sinus

mesenchyme (UGM) induces urogenital sinus epithelium to undergo prostatic ductal morphogenesis and differentiation. (2) As prostatic epithelium differentiates, it in turn signals the UGM to differentiate into smooth muscle cells that closely surround the epithelial ducts. Differentiation of prostatic smooth muscle occurs after an inductive signal from epithelium and androgens. (3) Once formed, prostatic smooth muscle participates in reciprocal homeostatic interactions.

Prostatic smooth muscle, under the influence of androgens, signals prostatic epithelium to maintain epithelial differentiation and to repress epithelial proliferation, while prostatic epithelium signals to prostatic smooth muscle to maintain smooth muscle differentiation. In adulthood, homeostasis is maintained through reciprocal interactions between smooth muscle and epithelial cells, with minimal proliferation of either cell type. Prostatic carcinogenesis, which is initiated following, *e.g.*, genetic damage to prostatic epithelium, involves a sequential disruption in these reciprocal homeostatic interactions with ensuing de-differentiation of both the emerging prostatic carcinoma cells and smooth muscle. Thus, following genetic insult to prostatic epithelium, the epithelium fails to signal appropriately to the smooth muscle, which then begins to de-differentiate. As smooth muscle differentiation begins to deviate, signaling from prostatic smooth muscle to prostatic epithelium becomes anomalous, resulting in progressive loss of control over epithelial differentiation and proliferation. During progression of prostatic carcinogenesis a vicious cycle is established in which both prostatic epithelium and smooth muscle de-differentiate.

This sequence of events is based on the ontogeny of prostatic smooth muscle differentiation during development and by the fact that the amount of smooth muscle progressively diminishes in human prostatic adenocarcinomas during progression from low to high grade cancers. Finally, in experimental tissue recombinants in which various normal or neoplastic prostatic epithelia were grown in combination with embryonic rat UGM, only differentiated (non-neoplastic) epithelia were capable of inducing differentiation of prostatic smooth muscle in UGM. Moreover, induced differentiation of tumor cells reduced tumorigenicity. *See*, the examples below. It is now apparent that smooth muscle-epithelial interactions are the operative cell-cell interaction in the postnatal prostate which plays a key role in regulating epithelial differentiation, proliferation and carcinogenesis.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. One of skill will recognize a variety of parameters which can be changed or modified to yield essentially similar results.

The following methods and materials were used in the Examples below.

Animals

Neonatal Sprague-Dawley rats and adult athymic mice were obtained from Simonson, Gilroy, CA. C57BL/6J mice were obtained from Jackson labs. Neonatal male rats and female mice were used within 24 hours of birth (day 0). All animals received water and laboratory chow *ad libitum* and were housed under standard laboratory conditions.

Antibodies

The polyclonal rabbit antibodies (IgG fraction) monospecific for the androgen-dependent proteins (proteins I-V) of rat SVS proteins have been described (Fawell, S.E. *et al. Mol. Cell. Endocrinol.* (1987) **50**:107-114). The antibody to mouse SVS proteins has been described (Higgins, S.J. *et al. Development* (1989) **106**:219-234). Higgins and co-workers have described all the immunocytochemical reagents and their sources (Higgins, S.J. *et al. Development* (1989) 106: 235-250; Higgins, S.J. *et al. Development* (1989) **106**:219-234).

Preparation of tissue recombinants

Seminal vesicles were excised from 0-day-old neonatal rats. Vaginas were excised from 0-day-old neonatal mice. Mesenchymes were separated from epithelium following tryptic digestion as described earlier (Cunha, 0.R. *et al. Human Genetics* (1981) **58**:68-77).

Following overnight culture of the rat SVM on a 1% agar substrate, the mesenchyme rounded up into a cohesive mass of tissue which was transplanted into adult male athymic mouse hosts anesthetized with Avertin (tertiary amyl alcohol, plus tribromoethanol). The transplant site for the SVM was the severed proximal end of the ureter of the adult male athymic mouse host. The grafted SVM was fixed in place by a thin platinum wire which acted as a staple, holding the neonatal rat SVM in contact with the adult mouse ureter.

The transplant site for the mouse vaginal mesenchyme was the bladder of the adult male C57BL/6J mouse. Bladders were exteriorized through a small, midventral

incision in the body wall. The bladder mucosa was exposed by an oblique incision in the muscular wall of the bladder. The vaginal mesenchyme was then inserted into the incision such that the neonatal mouse mesenchyme was in close proximity to the adult mouse bladder epithelium.

Recovery and processing of tissue recombinants

Hosts were killed 4-12 weeks after grafting. The grafts were isolated by dissection. Secretions were recovered from the lumina of the recombinants, solubilized in SDS, and stored as described (Higgins, S.J. *et al. Development* (1989) **106**:219-234). The remaining tissue was fixed by immersion overnight in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and air-dried onto poly-L-lysine-coated slides (Higgins, S.J. *et al. Development* (1989) **106**:219-234).

Immunocytochemistry

Antibodies against mouse SVS proteins and rat SVS protein IV were used for immunocytochemistry. The antibodies against mouse SVS proteins react with mouse but not rat SVS proteins, while antibodies against rat SVS protein IV reacts with rat but not mouse SVS proteins. The immunocytochemical methodology has been described in detail earlier (Higgins, S.J. *et al. Development* (1989) **106**:219-234; Prins, G. *et al. Endocrinology* (1991) **129**:3187-3199). Briefly, deparaffinized tissue sections were incubated successively with the primary antisera, biotinylated donkey anti-rabbit IgG, PBS-Tween, and the Vectastain peroxidase ABC reagent. The colored reaction product is then developed using diaminobenzidine and H_2O_2 using standard techniques.

Polyacrylamide gel electrophoresis

Protein samples prepared in PBS-SDS were analyzed by electrophoresis in polyacrylamide (10-20% linear gradient) slab gels using a discontinuous buffer system (Laemmli, U.K. *Nature* (1970) **227**:680-685) with 0.1% SDS throughout (Brooks, D.E. *et al. J. Reprod. Fertil.* (1980) **59**:363-375). Proteins (20-50 μg per lane) resolved by this method (SDS-PAGE) were visualized by staining for 1 hour in 0.1% Coomassie blue in acetic acid:methanol:H_2O (1:3:6 by vol) followed by prolonged destaining in the same solution without the dye.

Androgen Receptors

The PG21 antibody was used to detect androgen receptors in frozen tissue sections as described previously (Prins, G. *et al. Endocrinology* (1991) **129**:3187-3199).

The tissue sections were then stained using the Biotin/Avidin system (Vectastain kit, Vector Laboratories).

Example 1: Induction of Terminally Differentiated Adult Mouse Ureters

Left ureters of anesthetized adult male athymic mouse hosts were severed below the kidney and neonatal rat SVM was grafted to the cut end of the ureters, thus bringing adult mouse URE in contact with neonatal rat SVM (Fig. 1). The mesenchyme was held in place with a thin platinum wire. The hosts were killed four to eight weeks after grafting, and the *in situ* tissue recombinants were harvested and the secretions recovered. In 5 of 11, tissue recombinants were harvested and the secretions recovered. In 5 of 11 tissue recombinants, an instructive induction occurred, resulting in the *in situ* transformation of the adult non-glandular mouse urothelium into mouse SV tissue. The failure of induction to occur in 6 of the 11 cases is attributed to the detachment of the neonatal rat SVM from the graft site, as the platinum wire was found some distance from the ureter when the hosts were sacrificed. This detachment of the rat SVM from the graft site is not unexpected, because the surgery was difficult due to the small sizes of the tissues involved.

The tissue recombinants composed of neonatal rat SVM and 60-day-old (adult) nude mouse URE formed histologically recognizable SV tissue. Immunocytochemistry was performed with species-specific antibodies which showed that the induced SVE expressed mouse (not rat) SVS proteins. The presence of mouse SVS proteins in the induced glandular tissue was confirmed by polyacrylamide-gel electrophoresis (SDS-PAGE). Particularly evident in the secretions obtained from the induced glandular tissue were the low molecular weight triplet and protein #3 at about 27 kD which are characteristic of mouse SV secretions. 2 higher molecular weight proteins running at 30 and 66 kD were also present, but at lower levels.

In addition, a control was performed in which neonatal rat SV (both epithelium and mesenchyme) was grafted to the adult mouse ureter. In this case the secretions obtained were characteristic of rat (not mouse) SV.

Further evidence regarding the instructively induced glandular tissue in rat SVM + mouse URE tissue recombinants was obtained by nuclear staining with Hoechst dye 33258 (Cunha, G.R. *et al. Stain Technol.* (1984) **59**:7-12). Mouse nuclei stained with this dye display many intensely fluorescent spots, whereas rat nuclei stain homogeneously.

Use of this dye on rat SVM + mouse URE recombinants indicated that the induced SV tissue was mouse in origin (Fig. 6 and inset).

One feature of SVE, which is not shared with URE, is the expression of androgen receptors (AR). Neither epithelial nor stromal cells of the ureter exhibit AR. By contrast, AR are a prominent feature in both the epithelium and stroma of the SV. When adult URE was induced by SVM to differentiate into SV tissue, the induced epithelium expressed nuclear staining with the PG21 antibody to the AR.

As controls, rat bladder mesenchyme was transplanted to the severed ends of the ureters of adult male athymic mice as described above. In these tissue recombinants, the urothelial phenotype was maintained, and no SVS proteins were detected.

In addition, neonatal mouse vaginal mesenchyme was grafted beneath bladder mucosa of adult male mice, and the host animals were killed 3 months later. Such vaginal mesenchyme has been shown to be able to induce prostatic development when experimental tissue recombinants are grown in male hosts (Cunha, G.R. *Endocrinology* (1975) 95: 665-673). The vaginal mesenchyme implanted into tho bladders induced prostate like acini, indicating that the above reprogramming of adult organs (mouse URE by rat SVM) *in situ* is not an isolated occurrence.

Mesenchyme induced changes in adult epithelial differentiation described herein were examined from several standpoints: Gross morphological organization; epithelial cytodifferentiation; and expression of AR and tissue-specific secretory proteins. The glandular tissue induced by rat SVM in adult mouse URE *in situ* demonstrated a radical change in epithelial cytodifferentiation. The original transitional phenotype characteristic of a urothelium, which lacked AR (Cunha, G.R. *et al. Endocrinology* (1980) 107: 1767-1770), was transformed to a simple columnar secretory epithelium, which expressed both SVS proteins. It is likely that the expression of AR preceded, and is a prerequisite for, the production of SVS proteins as demonstrated previously (Cunha, G.R. *et al. Development* (1991) **111**:145-158). In the normal course of SV development, epithelial AR appear 2 to 3 days postpartum (Cooke, P.S. Endocrinol. Suppl. (1988) **122**:92; Shima, H. *et al. Endocrinology* (1990) **127**:3222-3233), whereas SVS protein synthesis begins after day 10 (Fawell, S.E. *et al. Mol. Cell. Endocrinol.* (1986) **48**:39-49).

The inductions described in this paper represent the first examples of complete morphological and functional reprogramming in adult epithelial cells by

transplantation of an inductive mesenchyme *in situ*. In essence, an animal was created with three seminal vesicles. This, obviously, has many clinical implications. Similar to the creation of a seminal vesicle, other organs are created utilizing instructive induction, *e.g.*, to assume organ function following natural organ failure. For instance, generation of lung, pancreas, liver, and kidney tissues are now possible *in situ*. For instance, a kidney can be induced *in vivo* by transplanting human metanephric mesoderm to the patient's own ureter. The urinary outflow tract is thus placed in an anatomically correct sequence relative to the developing kidney. Competent human metanephric mesoderm is found in embryos around embryonic day 35.

Example 2: Characterization of a Novel, Heterospecific Model of Prostate Development

A novel model of prostate development was developed which utilized tissue recombinants of adult human prostate epithelial cells (HPRE) and rat fetal urogenital sinus mesenchyme (UGM). The recombinants are grafted into adult male nude mouse hosts and the identity of the epithelial cells confirmed by species-specific *in situ* hybridization. The grafts underwent marked stromal and epithelial proliferation and ductal branching morphogenesis in an orderly fashion. Over the first 4 weeks, the epithelial cells reorganized into branching cords, which subsequently canalized to form ducts with a multilayered epithelium which expressed PSA and PAP. At this stage, all of the epithelial cells expressed both luminal and basal cytokeratins, a pattern characteristic of normal human fetal prostatic development. The ducts were surrounded by a thick, organized stroma which was rat in origin, but which had morphology characteristic of human prostate. Mitosis and apoptosis were prominent in both compartments during this phase of ductal-acinar growth. By 13 weeks, the epithelium underwent partial organization into luminal and basal compartments. The rate of development of the prostate recombinants was consistent with human, but not rodent prostate development. This demonstrated that HPRE is receptive to an inductive stroma of rodent origin and implies that different species share common mediators of stromal→epithelial interactions. Furthermore, there was evidence that the HPRE causes an altered stromal morphology in the rat UGM. This indicates the existence of uniquely human epithelial→stromal factor(s) which may be responsible for the predominance of stroma in the human prostate. This model is useful for the study of the stromal-epithelial interactions involved in human prostate development and BPH.

Example 3: Analysis Of Heterospecific Prostatic Tissue Recombinants

To examine more closely the interactions occurring between developing human prostate epithelium and inductive mesenchyme, the *in vivo* heterospecific tissue recombination model of Example 2 was used. This method uses rat urogenital sinus mesenchyme (rUGM) recombined with fresh adult human prostate epithelium (hPrE) in collagen gels grafted beneath the renal capsule of athymic mouse hosts. The resultant hPRE/rUGM tissue recombinants, when grafted into male athymic nude mice or rats, exhibit extensive epithelial growth and ductal branching morphogenesis. The epithelium initially forms solid cords which grow and branch into the surrounding mesenchyme. The solid epithelial cords then canalize, and the epithelium differentiates into basal and luminal cell types each expressing their characteristic cytokeratins. The human prostatic secretory proteins, prostatic acid phosphatase and PSA are then expressed in the luminal epithelial cells. Concurrent with this epithelial differentiation, the rat UGM differentiates into AR-expressing smooth muscle. The role of epithelium in dictating the pattern of stromal differentiation is underlined by the observation that the smooth muscle differentiates into thick sheets, thus exhibiting a pattern normally found in the human prostate as opposed to the thin sheathes characteristic of the rat from which the UGM was derived. These observations in human-rat heterospecific tissue recombinants parallel those seen in recombinations of rodent UGM with adult rodent prostatic ductal tips. However, in the case of a rodent/rodent tissue recombinant the process of growth and differentiation is much more rapid than in tissue recombinants containing human tissue. It is a measure of the flexibility of these models that tissue recombinants constructed with epithelium and mesenchyme from widely separated species exhibit both instructive and permissive inductions. This indicates that signaling between epithelium and mesenchyme uses the same language in the rodent and human, since a rodent mesenchyme induced prostatic differentiation in a human epithelium.

The induction of new prostatic ductal growth by rat UGM in adult human prostatic epithelium also substantiates the idea that benign prostatic hyperplasia is due to a reawakening of inductive potential in stroma of the adult prostate, by clearly demonstrating that adult human prostatic epithelium is responsive to the morphogenetic and growth promoting effects of a prostatic inducer.

The chimeric rat-human prostate described above lends itself to analysis of gene expression on a species specific basis. The epithelial component of the

heterospecific tissue recombinant is of human origin, the mesenchyme is rat, while the mouse host contributes the vasculature. Growth factors and their receptors are highly conserved between species so in most cases techniques such as Western and Northern blotting did not differentiate between proteins and mRNA derived from different species. However, other techniques such as RNase protection assays are able to distinguish messages by species of origin. As a screening technique to examine growth factor expression in heterotypic tissue recombinants, species specific RT-PCR assays for a number of growth factors and their receptors were developed. These assays work on the principle that growth factors are highly conserved between species. It was possible to develop PCR primer sets which amplify a cDNA fragment from all three species of interest (*e.g.*, human, rat and mouse). Typically, these fragments are virtually the same and are not resolved by gel electrophoresis. However, careful analysis of the nucleotide sequences of the amplified fragments reveals small (often conservative) differences which allow for species specific digestion with restriction endonucleases, thereby permitting simplified screening.

For most growth factors, which have highly conserved mRNA sequences between species, a single set of RT-PCR primers can amplify a given sequence for multiple species. The amplified fragments of such a reaction were virtually the same size and not distinguishable by gel electrophoresis. However, due to minor variability in their nucleotide sequences, amplified fragments from each species contained unique restriction endonuclease cleavage sites. The amplified fragments were distinguished by restriction digestion of the amplified fragment followed by gel electrophoresis of the products of the digestion. Thus, by using SS-RT-PCR, heterospecific tissue recombinants were analysed to determine the species (and thus the tissue type) producing the mRNA under investigation. For example, a set of PCR primers which amplify TGF-β3 cDNA from human, rat or mouse were developed. These primers produced an amplified fragment which contained restriction endonuclease sites which are unique for each species, such that specific digestions can be used to identify the species of origin (and thus tissue layer) of the signal. Detail of the primer sequences, digestion enzymes and product sizes are shown below. In this tissue recombinant, digestion with the enzymes Dde 1 and Msp 1, which digest human and mouse product, respectively, leads to a small amount of species specific digestion product. The enzyme Bsp12861 digests the amplified product from all three species; however, it produced a 252bp fragment which is unique to the rat.

Digestion with this enzyme shows that the 252bp (rat specific) fragment is the principle digestion product. The data presented here show that TGF-β3 is expressed in human, rat and mouse tissue, suggesting that this growth factor is expressed in epithelium, stroma and the vasculature of the graft. While the method is not quantitative, the data suggested that most of the signal is produced in the stromal portion of the graft. Confirmation is obtained by using RNAse protection assays which are both quantitative and species specific. SS-RT-PCR is used to analyze the expression of growth factors and growth factor receptors in the interacting epithelium and stroma of rat UGM + human prostatic epithelium and stroma of rat UGM + human prostatic epithelium tissue recombinants growing *in vivo*.

Smooth muscle plays a critical role in prostatic carcinogenesis. From a biological viewpoint, this hypothesis integrates developmental biology with the carcinogenetic process, which has been suggested previously to represent a caricature of differentiation. Carcinogenesis is a disease of tissue differentiation and, therefore, carcinogenesis is an evolutionary process regulated and influenced at every step by cell-cell interactions. Thus, while mesenchymal-epithelial and smooth muscle-epithelial interactions are the basis of normal development and adult tissue homeostasis, regulatory interactions between epithelium and smooth muscle cells become progressively decreased during promotion and progression of prostatic carcinomas.

In addition to integrating normal development and cancer, this discovery provides the basis of novel therapeutic approaches to neoplasms, *i.e.*, differentiation

therapy, whereby carcinoma cells are induced to differentiate with a concomitant reduction on growth rate.

Primer Set	5' primer sequence	3' primer sequence
TGFβ3	TGCCCAACCCCAGCTCTAAGCG	GCCTTTGAATTTGATTTCCA

Species amplified	5' primer	3' primer	PCR product	Enzyme	Digested fragments
human	729-750	999-1018	288 bp	DdeI	172, 103, 11 bp
rat	865-886	1135-1154	288 bp	Bsp12861	252, 38 bp
mouse	1071-1092	1341-1360	288 bp	MspI	231, 57 bp

Primer sets, restriction digestions and product sizes for TGF-β3 expression in a human prostate epithelium/rat urogenital sinus mesenchyme tissue recombinant.

Expression of muscle markers in the developing rat anterior prostate

	19dE UGS	Od postnatal	5d postnatal	10d postnatal	15d postnatal	Adult
Actin	++	++	++	+++	+++	+++
Myosin	-	-	+	+	++	+++
Desmin	-	-	+/-	+	+	+++
Laminin	-	-	-	+/-	+	+++
Vinculin	+/-	+	++	++	++	+++
Vimentin	++	++	++	++	++	+

Vimentin is initially widely expressed and during development becomes localized to the interducial connective tissue and excluded from the differentiating muscle. The markers of smooth muscle differentiation are expressed sequentially, in a proximal to distal manner along the growing ducts.

Example 4: Smooth Muscle Regulates Prostatic Homeostasis And Malignant Growth

While mesenchymal-epithelial interaction is a term used to describe morphogenetic cell-cell interactions during development, the term stromal-epithelial interaction is used for such cell-cell interactions in adulthood. Stroma is a term that denotes the non-epithelial component of an organ. For most internal organs, the

predominant cell types in stroma are fibroblasts and smooth muscle. The principle stromal interaction of adult prostatic epithelium is with the surrounding smooth muscle. In the rodent prostate, smooth muscle cells are organized into thin sleeves of 3 to 4 layers of AR-positive smooth muscle cells interspersed with a few AR-negative fibroblasts. The composition and relative amount of smooth muscle and fibroblasts surrounding the epithelial ducts varies proximal-distally from the urethra out to the ductal tips. Proximally near the urethra, the stromal sheath is thickest and predominantly of smooth muscle. At the ductal tips distally, the stromal sheath is formed of a discontinuous layer of smooth muscle cells with interspersed fibroblasts. In the so-called intermediate zone, the stromal sheath is composed of a thin continuous layer of smooth muscle cells. Prostatic smooth muscle cells are in intimate contact with the basement membranes of epithelial ducts as epithelial and smooth muscle cells are separated by only about 300nm. Given the close proximity of AR-positive smooth muscle cells with prostatic epithelium, interaction of adult prostatic epithelium with its immediate stromal environment is generally a smooth muscle-epithelial interaction even though the "acinar capsule" surrounding individual epithelial ducts also contains a small number of fibroblasts.

Since AR-positive smooth muscle constitutes about 22% of the human prostate, a major cell type in cultures of "prostatic stroma" is, in fact, de-differentiated smooth muscle cells. Indeed, prostatic stroma of the guinea pig contains only 5% fibroblasts, with smooth muscle being the major cell type. This is not easily recognized in cell culture, because smooth muscle cells are known to rapidly dedifferentiate into fibroblast-like cells when grown *in vitro*. Once prostatic smooth muscle differentiates from UGM, the smooth muscle cell becomes an interactive element in the epithelial micro-environment and in homeostatic cell-cell interactions in the adult prostate.

In adulthood, normal prostatic epithelial growth and differentiation are regulated by reciprocal smooth muscle-epithelial cell interactions mediated by the local synthesis and action of growth factors and other paracrine signaling molecules.

These observations integrate the role of cell-cell interactions in normal development and carcinogenesis of the prostate and are also based on the following facts. UGM induces prostatic ductal morphogenesis and epithelial differentiation, and developing prostatic epithelium in turn induces smooth muscle differentiation in UGM. After differentiation of prostatic smooth muscle, reciprocal smooth muscle-epithelial interactions play a homeostatic role in maintaining prostatic structure and function. Expressly, smooth

muscle is proposed to maintain prostatic epithelial structure and function, while prostatic epithelium in turn maintains smooth muscle differentiation. As a result of genetic damage to prostatic epithelium, the initiated epithelium begins to diverge from its normal phenotype, and thus signaling from prostatic epithelial cell to smooth muscle becomes aberrant. As a consequence of altered epithelial-smooth muscle signaling, prostatic smooth muscle cells begin to de-differentiate, resulting in abnormal smooth muscle→epithelial signaling. Abnormalities in smooth muscle→epithelial signaling may either actively promote carcinogenesis or permit the progression to anaplasia through the loss or restriction of normal homeostatic/growth inhibitory controls. The result is a cycle of sequential loss of differentiation in both epithelial and smooth muscle compartments, leading to promotion and progression of the tumor, and ultimately culminating in clinical cancer.

Prostatic epithelium is an inducer of smooth muscle differentiation, while UGM induces prostatic epithelial differentiation. A prediction of this hypothesis is that normal (but not transformed) prostatic epithelium should be capable of inducing smooth muscle differentiation in UGM. This prediction was verified. Tissue recombinants were prepared with 18 day embryonic rat UGM associated with various normal and neoplastic prostatic epithelia. In all cases, when normal embryonic or adult prostatic epithelia were utilized, the resultant prostatic ductal structures were surrounded by compact sheathes of smooth muscle cells that developed from the UGM. Conversely, when anaplastic prostatic epithelia were used, smooth muscle failed to differentiate in the tissue recombinants, and instead the UGM maintained its mesenchymal nature. The one prostatic carcinoma cell line capable of inducing smooth muscle differentiations was Dunning tumor epithelium, which is a highly differentiated cell. Thus, normal prostatic epithelium can induce smooth muscle differentiation in UGM, while transformed prostatic epithelium cannot.

Another prediction of the hypothesis is that progression of human prostatic carcinoma from benign through low grade to high grade carcinoma should be associated with a successive loss of smooth muscle or a loss of smooth muscle differentiation markers. Based upon immunocytochemical staining for smooth muscle α-actin, normal and hyperplastic human prostates have substantial amounts of smooth muscle surrounding the epithelial ducts. Conversely, the amount of smooth muscle is notably reduced in prostatic neoplasia. This loss in smooth muscle was especially evident in close proximity

to prostatic carcinoma cells. Indeed, foci of prostatic carcinoma cells were surrounded by domains of fibroblastic stroma deficient in smooth muscle. Peripheral to these zones of fibroblastic cells were poorly organized sparse bundles of smooth muscle. Such observations support the idea that prostatic carcinoma cells are incapable of inducing and maintaining smooth muscle differentiation as indicated by the tissue recombinant experiments described above. It is noteworthy that a feature of the Dunning prostatic adenocarcinoma is the absence of smooth muscle in association with the aberrant epithelial ducts. Thus, the process of prostatic carcinogenesis entails aberrations in the interactions of the prostatic epithelium with its smooth muscle micro-environment resulting in reciprocal dedifferentiation of both the emerging carcinoma cells as well as prostatic smooth muscle. Such alterations in cell-cell signaling affect differentiation and growth control of prostatic epithelium as it becomes progressively anaplastic, but also involves a fundamental change in stroma, as prostatic smooth muscle is replaced by a fibroblastic stroma that in turn facilitates epithelial anaplasia.

These results are consistent with the concept of tumor stroma as an essential component of the carcinogenic process. The dynamic interaction of emerging or established carcinoma cells with stroma lead to the concept that stroma is not an inert scaffold supporting the epithelial neoplasm, but instead is an active, essential player in carcinogenesis and tumorigenesis. Stromal cell alterations have been reported for many carcinomas. While many of the "stromal changes" associated with carcinogenesis may be related to bona fide fibroblasts, smooth muscle cells constitute a significant proportion of the fibromuscular stroma of certain organs such as the prostate and thus, stromal changes during carcinogenesis involve smooth muscle cells as well. One of the stromal changes associated with prostatic carcinogenesis is increased agglutinability by concanavalin A of human peritumoral prostatic fibroblasts. In so far as this observation is based upon analysis of cultured prostatic stromal cells, it may be that the so-called prostatic fibroblasts described are in fact de-differentiated smooth muscle cells which in culture resemble fibroblasts. In any case, the carcinogenetic process appears to encompass progressive and reciprocal changes in both epithelial and stromal/smooth muscle components.

A corollary of cell-cell interactions in the prostate is that for some prostatic adenocarcinomas it is possible to reconstitute a more regular tissue architecture and a slower growth rate through interaction of the carcinoma cell with normal stroma. Stromal-epithelial interactions in the Dunning prostatic adenocarcinoma (DT) are clearly

abnormal, as the basement membrane interposed between the neoplastic epithelia ducts and the "stroma" is frequently discontinuous and/or multi-layered. This observation raised the possibility that growth and differentiation of the DT epithelial cells might be modified if these cells could be reassociated with a more normal "stromal" environment. To test this possibility, 0.5 mm^3 fragments of the DT were grown, for one month in association with UGM, bulbourethral gland (BUGM), or seminal vesicle mesenchyme (SVM) in male nude mouse hosts. Grafts of DT alone maintained the histopathology distinctive of the DT and formed tumors containing small ducts lined by one to two layers of undifferentiated squamous or cuboidal epithelial cells. In grafts of UGM + DT, BUGM + DT, or SVM + DT, the mesenchyme induced the undifferentiated DT epithelial cells to differentiate into tall columnar secretory epithelial cells assembled into large cystic ducts. These mesenchyme-induced changes in DT histodifferentiation were associated with changes in neoplastic growth. When SVM-induced differentiated DT cells were tested for their ability to grow as tumors, tumorigenesis was remarkably diminished. For example, when ducts of SVM+DT recombinants were isolated and grafted to new male hosts or combined with fresh SVM to form secondary SVM+DT recombinants, overall growth was minimal during a 3 month period, and the highly differentiated state of the DT epithelium was maintained. In contrast, grafts of the DT itself overgrew the host's kidney, producing a single large tumor mass weighing 5 to 7 grams. The reduction in growth and extreme reduction in tumorigenesis of the SVM-induced DT epithelial cells was associated with a 7-fold reduction in ^{3}H-thymidine epithelial labeling index. Significantly, smooth muscle cells evidently derived from the SVM had differentiated in SVM+DT tissue recombinants and were in close apposition to the highly differentiated DT epithelium.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, modifications can be made thereto without departing from the spirit or scope of the appended claims.

All publications and patent applications cited in this specification are herein incorporated by reference for all purposes as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

WHAT IS CLAIMED IS:

1. A method of producing a differentiated cell with a selected phenotype comprising:

(i) providing a mesenchymal cell;

(ii) contacting said mesenchymal cell with a second cell; and,

(iii) incubating the mesenchymal cell with the second cell, thereby inducing the second cell to produce the differentiated cell with the selected phenotype.

2. The method of claim 1, wherein the second cell is an epithelial cell.

3. The method of claim 1, wherein step (iii) is performed *in vivo*.

4. The method of claim 1, wherein step (iii) is performed *in vitro*.

5. The method of claim 1, wherein step (iii) is performed *in vitro*, and wherein the method further comprises introducing the differentiated cell into an animal.

6. The method of claim 1, wherein the mesenchymal cell and the second cell are from organisms of different species.

7. The method of claim 1, wherein the mesenchymal cell is isolated from tissue which comprises cells with the same phenotype as the selected phenotype.

8. The method of claim 7, wherein the second cell and the mesenchymal cell are both derived from the same organ.

9. The method of claim 7, wherein the second cell and the mesenchymal cell are derived from different organs.

10. The method of claim 1, wherein a plurality of differentiated cells are produced, thereby forming a tissue.

11. The method of claim 1, wherein the mesenchymal cell gives rise to differentiated tissue during the development of the organism, wherein the differentiated tissue has the same phenotype as the selected phenotype.

12. The method of claim 1, wherein a plurality of differentiated cells are produced, thereby forming a tissue, wherein the tissue is selected from the group of tissues consisting of kidney tissue, pancreatic tissue, bladder tissue, lung tissue, intestinal tissue, liver tissue, prostate tissue and reproductive tissue.

13. The method of claim 1, wherein a plurality of differentiated cells are produced, thereby producing epithelial tissues.

14. The method of claim 13, wherein said epithelial tissue is selected from the group consisting of kidney tissue, pancreatic tissue, bladder tissue, lung tissue, intestinal tissue, liver tissue, prostate tissue and reproductive tissue.

15. The method of claim 1, wherein the second cell is selected from the group of cells consisting of a tumor cell and an immortalized cell.

16. The method of claim 15, wherein the differentiated cell is not a tumor cell.

17. A method of producing a selected cell from a terminally differentiated cell, the method comprising incubating said terminally differentiated cell with a mesenchymal cell.

18. The method of claim 17, wherein said terminally differentiated cell is an epithelial cell.

19. The method of claim 17, wherein said terminally differentiated cell is an epithelial cell derived from a tissue selected from the group consisting of kidney tissue, pancreatic tissue, bladder tissue, lung tissue, intestinal tissue, liver tissue, prostate tissue and reproductive tissue.

20. The method of claim 17, wherein the terminally differentiated cell is a ureter cell, and the selected cell is a kidney cell.

21. The method of claim 17, wherein the selected cell is present in an organ.

22. The method of claim 17, wherein the selected cell is present in an organ selected from the group consisting of seminal vesical, kidney tissue, pancreatic tissue, bladder tissue, lung tissue, intestinal tissue, liver tissue, and reproductive tissue.

23. A kit comprising mesenchymal cells and a container.

24. The kit of claim 23 which further comprises instructional materials.

25. A cell produced by incubating a terminally differentiated cell with an embryonic mesenchymal cell.

26. The cell of claim 25, wherein said cell is present in an animal.

27. A mesenchymal-epithelial tissue recombinant comprising a host animal of a first species, a mesenchymal cell from a second species and an epithelial cell from a third species.

28. The mesenchymal-epithelial tissue recombinant of claim 27, wherein the host animal is a mouse, the mesenchymal cell is from a rat embryo and the epithelial cell is a human cell.

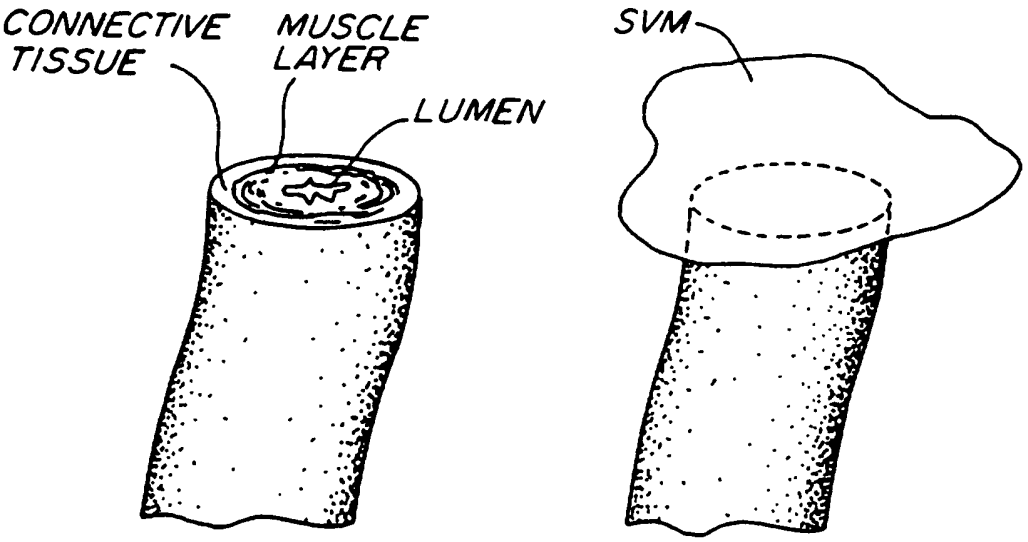

FIG. 1.

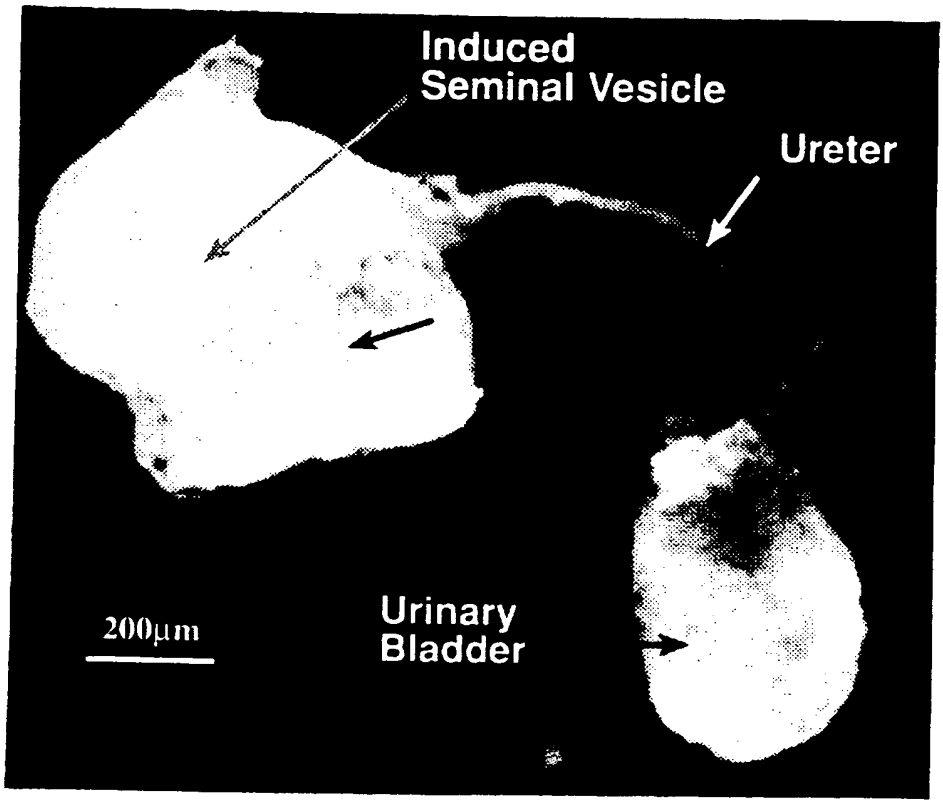

FIG. 2.

INTERNATIONAL SEARCH REPORT

Inter. ..ional application No.
PCT/US96/14781

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/85; A61K 48/00
US CL :435/240.2; 424/93.21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2; 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	LIPSCHUTZ et al. Urothelial transformation into functional glandular tissue in situ by instructive mesenchymal induction. Kidney International. January 1996, Vol. 49, pages 59-66, see entire document.	1-28
Y	DONJACOUR et al. Induction of prostatic morphology and secretion in urothelium by seminal vesicle mesenchyme. Development. July 1995, Vol. 121, No. 7, pages 2199-2207, see entire document.	1-28
Y	CUNHA et al. Mammary Phenotypic Expression Induced in Epidermal Cells by Embryonic Mammary Mesenchyme. Acta Anat. 1995, Vol. 152, pages 195-204, see entire document.	1-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents.	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 NOVEMBER 1996	Date of mailing of the international search report 03 DEC 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer D. CURTIS HOGUE, JR. Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

Inter...tional application No.
PCT/US96/14781

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HAYASHI et al. Permissive and Instructive Induction of Adult Rodent Prostatic Epithelium by Heterotypic Urogenital Sinus Mesenchyme. Epithelial Cell Biology. 1993, Vol. 2, pages 66-78, see entire document.	1-28
Y	CUNHA et al. Developmental Response of Adult Mammary Epithelial Cells to Various Fetal and Neonatal Mesenchymes. Epithelial Cell Biology. 1992, Vol. 1, pages 105-118, see entire document.	1-28
Y	TSUJI et al. Effect of Mesenchymal Glandular Inductors on the Growth and Cytodifferentiation of Neonatal Mouse Seminal Vesicle Epithelium. Journal of Andrology. November/December 1994, Vol. 15, No. 6, pages 565-574, see entire document.	1-28